FORM PTO-1390 (REV 11-98)

U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **MESSIKA=2**

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/445105

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/IL98/00254	**01 June 1998**	**02 June 1997**

TITLE OF INVENTION: **PREPARATION OF GLYCOSYLATED TUMOR NECROSIS FACTOR**

APPLICANT(S) FOR DO/EO/US: **Ziva MESSIKA et al.**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

    1. **A courtesy copy of the specification as originally filed.**
    2. **A courtesy copy of the first page of the International Publication (WO98/55622).**
    3. **A courtesy copy of the International Search Report.**
    4. **A courtesy copy of the International Preliminary Examination Report.**
    5. **Sequence Listing diskette.**
    6. **Formal drawings, 7 sheets, figures 1-2.**

Rec'd PCT/PTO 02 DEC 1999

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/445105	PCT/IL98/00254	MESSIKA=2

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

		CALCULATIONS PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	$970.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	$840.00	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$760.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$670.00	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	$96.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		$ 840.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		$ 130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	6 - 20 =		X $18.00	$	
Independent claims	4 - 3 =	1	X $78.00	$	78.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $260.00	$	
TOTAL OF ABOVE CALCULATIONS =				$	1,048.00
Reduction of 1/2 for filing by small entity, if applicable. A Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$	1,048.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	1,048.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$	1,048.00
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ **1,048.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-4035**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

BROWDY AND NEIMARK, P.L.L.C.
419 Seventh Street N.W., Suite 300
Washington, D.C. 20004

SIGNATURE

Roger L. Browdy
NAME

25,618
REGISTRATION NUMBER

Date of this submission: December 2, 1999

Please substitute the attached Sequence Listing (1 page) for the Sequence Listing as originally filed in the parent application.

REMARKS

The above amendment to the specification is being made to insert reference to the PCT application of which the present case is a U.S. national stage.

Applicants have added into the present specification a substitute paper copy Sequence Listing section according to 37 C.F.R. §1.821(c) and §1.823(a). Furthermore, attached hereto is a 3 1/2" floppy disk containing the "Sequence Listing" in computer readable form in accordance with 37 C.F.R. §1.821(e).

The following statement is provided to meet the requirements of 37 C.F.R. 1.825(a) and 1.825(b).

I hereby state, in accordance with 37 C.F.R. §1.825(a), that the amendments included in the substitute sheets of the sequence listing are believed to be supported in the application as filed and that the substitute sheets of the sequence listing are not believed to include new matter.

I hereby further state, in accordance with 37 C.F.R. §1.825(b), that, on information and belief, the attached copy of the computer readable form is the same as the attached substitute paper copy of the sequence listing.

Applicants submit that the present application contains patentable subject matter and therefore urge the examiner to pass the case to issuance.

If the examiner has any questions or comments concerning the above described application, the examiner is urged to contact the undersigned at the phone number below.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

By _____
Roger L. Browdy
Registration No. 25,618

RLB:bcs
419 Seventh Street, N.W.
Washington, D.C. 20004
Telephone No.: (202) 628-5197
Facsimile No.: (202) 737-3528
f:\user3\99nov\mes2.pa

SEQUENCE LISTING

<110> MESSIKA, Ziva
HAREL, Adrian
ZE'EVI, Menachem

<120> PREPARATION OF GLYCOSYLATED TUMOR NECROSIS FACTOR

<130> messika2

<140> 00

<141> 1999-12-02

<150> PCT/IL98/00254

<151> 1998-06-01

<150> 120979

<151> 1997-06-02

<160> 1

<170> PatentIn Ver. 2.0

<210> 1

<211> 15

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial SequenceRecombinant
Human Gene formed using E. Coli and CHO cells

<400> 1

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
1 5 10 15

PREPARATION OF GLYCOSYLATED TUMOR NECROSIS FACTOR

Field of the Invention

The present invention relates to glycosylated human tumor necrosis factor-alpha (TNF-α), pharmaceutical composition containing it, and methods for its preparation and use.

Background of the Invention

Cytokines are polypeptide mediators secreted by cells that affect the function of other cells and which play an important role in the interactions between cells in the immune system. Together with other substances, cytokines constitute the molecular language of inflammation and immunity and form a complex of interacting and overlapping networks of signals that orchestrate the body's defensive reactions. One such cytokine is tumor necrosis factor (TNF).

TNF is one of a family of proteins that orchestrate the body's remarkably complex response to injury and infection. TNF is implicated in a wide variety of human diseases, may be effective against certain cancers, plays a role in septic shock and cachexia, is a mediator of inflammation in various immunological reactions, and also seems to be important in some pathological effects seen in malaria and meningococcal septicemia. With tumor necrosis factor, as with many other factors produced by the body, there seems to be a fine line between benefit and harm: an agent that is helpful in the local control of injury and infection may be toxic when it is released in large amounts or in the wrong place. See Old, "Tumor Necrosis Factor", Scientific American, 258:59-60, 69-75 (May 1988).

TNF exerts its effect by binding to specific receptors on cell membranes and causing clustering of the receptors. Two TNF receptors, with molecular weight of 55,000 Daltons and 75,000 Daltons, are known. After TNF binds to its receptor, the complex is internalized and degraded. Receptor clustering activates protein kinases

and phospholipases, leading to production of signal mediators and secondary messengers, resulting in cell death.

The TNF "family" includes two structurally and functionally related proteins, TNF-alpha and TNF-beta. The close relationship of these proteins was not known until 1984 when coding of the cDNAs for human TNF-alpha and TNF-beta, unexpectedly revealed that they are about 30% homologous at the amino acid level. Both TNF-alpha and TNF-beta bind to the same cell surface receptors, and they are very similar (though not identical) in the spectra of their activity. However, the regulation of expression of the TNF-alpha and the TNF-beta genes and the processing of the two corresponding proteins by the producing cells are completely different.

TNF-α has previously been referred to as cachectin and is produced primarily by mononuclear phagocytes, such as macrophages and monocytes. It is also produced by astrocytes, endothelial cells and some transformed cells. Mature human TNF-alpha is a polypeptide of 157 amino acid residues. The apparent molecular mass of human TNF-alpha is 17,000 Daltons. In its native form, TNF-alpha is a trimer. Among the indications for which TNF-α is known are melanoma, EL-4 lymphoma, Meth-A tumors, melanocytes, leukemia, carcinomas of the liver or lung, metastases or mammary carcinomas, cancers including breast, anal, colorectal, head, neck and other body organs. TNF has been found to have potential anti-microbial properties and is extremely potent against malarial and other parasites. TNF has been found to protect cells against some viral infections and prevent autoimmune diseases such as Type 1 diabetes. TNF inhibits lipid accumulation and alterations in adipose specific RNAs. TNF prevents the expression of genes responsible for producing enzymes important in storing fat. TNF exhibits a synergistic cytotoxic activity with interferon.

TNF-beta, also known as lymphotoxin and produced primarily by lymphocytes, contains 171 residues and has an apparent molecular mass of 25,000 Daltons. In addition to the 14 additional amino acids present at the amino terminus of TNF-beta, the difference in the size of the two molecules has been attributed to the absence of N-glycosylation in human TNF-alpha, as opposed to the presence of one

N-glycosylation site in TNF-beta. Vilcek et al, J.Biol.Chem. 266:7313-7316 (1991). Although murine TNF-alpha is a glycoprotein, there is no evidence to suggest that human TNF-alpha is glycosylated. Indeed it is described in the literature as being non-glycosylated (Markham et al, Eur. Cytokine Netw., 6: 49-54 (1995); Fiers, FEBS letters, 285:199-212 (1991)).

Human TNF-alpha can be isolated from human cells or prepared by recombinant techniques. While, theoretically, human TNF-alpha can be prepared by recombinant methods using any type of cells, conventionally TNF-alpha is prepared using transfected *E. coli*, cf. Wallace et al., U.S. Patent No. 4,879,226 and Aggarwal et al., EP 0168214. In fact, Aggarwal et al. specifically state that cultures of cells derived from multicellular organisms may be used as hosts, but that this is not preferred because of the excellent results obtained with TNF expressing microbes.

Subtle differences have been found between native human TNF-alpha and recombinant human TNF-alpha. The isoelectric point of native TNF-alpha is 5.6 and that of recombinant human TNF-alpha prepared in *E. coli* is 5.3.

Korn et al, Lymphokine Res., 7:349-358 (1988), reports the production of TNF in eukaryotic Chinese hamster ovary (CHO) cells and states that the advantage of a eukaryotic expression vector is a reduction of problems with endotoxin contamination as endotoxin may precipitate septic shock. The specific means of isolating the TNF actually used in this publication involved immunoprecipitation of the culture supernatant with polyclonal rabbit anti-TNF antibody with the immunoprecipitated material being released from SEPHAROSE beads by boiling in sample buffer containing SDS and beta-mercaptoethanol and run on a 15% SDS-polyacrylamide gel for two hours at 30 mA. Such a treatment would cause substantial loss of the biological activity of the protein. Thus, the actual product of the Korn publication is denatured and is not an isolated biologically active glygosylated TNF. Furthermore, Korn et al never recognized that the TNF produced by that method was glycosylated.

Marmenout et al, Eur. J. Biochem, 152:512-522 (1985), discloses the recombinant expression of TNF cDNA in monkey COS cells. While this publication reports expression of TNF activity in the culture medium, there is no disclosure that TNF was ever separated from the medium in any way. Indeed, there is no disclosure that it was even subjected to SDS-PAGE chromatography. Marmenout states that the available data suggests that human TNF is not glycosylated. Because of the substantially greater expression achieved in *E. coli* than from the COS cells, the remaining work reported in the Marmenout publication was done with the *E. coli* expression product, which is necessarily non-glycosylated.

Malik et al., in European Journal of Cancer, 26(1): 1031-1034 (1990), studied the action of TNF as a tumor-promoting agent by causing Chinese hamster ovary (CHO) cells to act as a tumor to demonstrate that TNF can stimulate tumor progression. In this case, the CHO cells transfected with the gene for human TNF were implanted directly into mice and the TNF produced by these cells was never isolated.

Summary of the Invention

Pure recombinant human TNF-alpha has now been produced in Chinese hamster ovary (CHO) cells and purified to homogeneity. This purified TNF-alpha has surprisingly been found to be glycosylated.

The pure recombinant human TNF-alpha produced in CHO cells was partially characterized by a variety of methods including capillary electrophoresis, carbohydrate composition, carbohydrate mapping, isoelectric focusing, SDS-PAGE, and RP-HPLC. These analyses indicated that recombinant human TNF-alpha produced in CHO cells was different from recombinant human TNF-alpha produced in *E. coli*.

Although human TNF-alpha is described in the literature as a non-glycosylated protein, the recombinant human TNF-alpha obtained from CHO cells, according to the present invention was found to contain multiple charged species.

When digested by neuraminidase, an enzyme that cleaves sialic acid, CHO recombinant human TNF-alpha multiple charged species collapsed to one major isoform/charged form as determined by isoelectric focusing and capillary electrophoresis, indicating that the variability of isoforms/charged forms stems from sialic acid and, hence, the molecule is glycosylated. Carbohydrate composition and mapping demonstrated the existence of O-glycosylation.

Neuraminidase and O-glycanase-digested CHO recombinant human TNF-alpha co-migrated with *E. coli* recombinant human TNF-alpha on SDS-PAGE, isoelectric focusing gels, and co-eluted on the capillary electrophoresis and reverse phase-high performance liquid chromatography (RP-HPLC), indicating that both proteins are similar in their polypeptide core but differ in the presence of O-linked oligosaccharide in the CHO recombinant human TNF-alpha.

The glycosylated human recombinant TNF prepared according to the present invention is particularly useful because, compared to non-glycosylated human TNF, the glycosylated TNF may have an increased half-life in body fluids, may improve binding to receptors, and may be better protected against the influence of proteases.

Brief Description of the Drawings

Figures 1A and 1B are oligomaps of CHO recombinant human TNF-alpha. Figure 1A shows the "O" mode, in which O-linked glycoside linkages are preferentially released, and Figure 1B shows the "N + O" mode, in which both N-linked and O-linked glycosidic linkages are released. The oligomaps indicate only the presence of one main O-linked oligoform.

Figures 2A-2E show capillary electrophoresis analysis of CHO recombinant human TNF-alpha. Figure 2A shows CHO recombinant h-TNF; Figure 2B shows CHO recombinant h-TNF plus neuraminidase; Figure 2C shows CHO recombinant h-TNF plus neuraminidase plus O-glycanase; Figure 2D shows CHO

recombinant h-TNF plus neuraminidase plus O-glycanase + N-glycanase. Figure 2E shows *E. coli* recombinant human TNF.

Detailed Description of the Invention

For the purpose of the present invention, tumor necrosis factor-alpha will be referred to as tumor necrosis factor or TNF and tumor necrosis factor-beta will be referred to as lymphotoxin.

In synthesizing glycosylated tumor necrosis factor, DNA which encodes tumor necrosis factor is ligated into a replicable (reproducible) vector, the vector used to transform host CHO cells, the CHO host cells are cultured, and TNF is recovered from the culture medium. This general process is used to construct glycosylated human TNF. The tumor necrosis factor species which are capable of synthesis herein include mature (valyl amino-terminal) tumor necrosis factor, pre-tumor necrosis factor ("pre-TNF", defined herein), and derivatives of TNF including fusion proteins wherein TNF (including mature tumor necrosis factor) is linked to other proteins or polypeptides by a peptide bond at the amino and/or carboxyl terminal amino acids to TNF. Human tumor necrosis factor synthesized in CHO recombinant culture is characterized by the presence of non-human cell components, including proteins, in amounts and of a character which are physiologically acceptable for administration to patients in concert with the tumor necrosis factor. These components are generally present in innocuous contaminant quantities, on the order of less than 1 percent by weight. Further, CHO recombinant cell culture enables the production of tumor necrosis factor absolutely free of homologous proteins.

Homologous proteins are those which are normally associated with the tumor necrosis factor as it is found in nature, e.g., in cells, cell exudates, or body fluids. For example, a homologous protein for human tumor necrosis factor is human serum albumin. Heterologous proteins are the converse, i.e., they are not naturally associated or found in combination with the tumor necrosis factor in question.

"Substantially homogeneous" glycosylated TNF means glycosylated TNF which is substantially free of other proteins native to the source from which the glycosylated TNF was isolated. This means that homogeneous glycosylated TNF is substantially free of blood plasma proteins such as albumin, fibrinogen, serine proteases, alpha- globulins, non-TNF cytotoxic polypeptides such as lymphotoxin or interferons, or other proteins of the cell or organism which serve as the synthetic origin of the glycosylated TNF, including whole cells and particulate cell debris.

Ordinarily, if a mammalian cell is transformed with (a) a vector containing the entire tumor necrosis factor structural gene (including a 5' start codon), or (b) the gene for mature tumor necrosis factor or a TNF derivative operably ligated to a secretory leader, which may also include the TNF secretory leader presequence, and the cell cultured, then mature TNF is recovered from the culture medium.

Similarly, if DNA which encodes TNF is operably ligated in a vector to a secretory leader which is properly processed by the host cell to be transformed, the host transformed with the vector and cultured then the tumor necrosis factor is synthesized without amino-terminal methionyl or blocked methionyl. Secretory leaders and host cells may be selected that also result in improper transport of mature protein into cell periplasm.

Once tumor necrosis factor is prepared by cell bioprocess, it generally is purified by recovering the supernatant culture fluid, removing solids, absorbing TNF from the supernatant admixture (containing TNF and other proteins) onto a hydrophobic substance, eluting TNF from the substance, adsorbing TNF onto a tertiary amino anion exchange resin, eluting TNF from the resin, adsorbing TNF onto an anion exchange resin (preferably quaternary amino-substituted) having substantially uniform particle size, and eluting TNF from the resin. The TNF composition can be optionally concentrated and purified by chromatofocusing at any point in the purification procedure, for example by isoelectric focusing or passage through a sieving gel such as SEPHADEX G-25.

The tumor necrosis factor obtained according to the present invention from transfected CHO cells is always obtained as a glycoprotein.

Purified glycosylated TNF from recombinant cell culture is combined for therapeutic use with pharmaceutically acceptable carriers and excipients, such as physiologically innocuous stabilizers and excipients, and prepared in dosage form as by lyophilization in dosage vials or storage in stabilized aqueous preparations. Alternatively, TNF may be incorporated into a polymer matrix for implantation into tumors or surgical sites from which tumors have been excised, thereby effecting a time-release of the tumor necrosis factor in a localized high gradient concentration.

In therapeutic applications, glycosylated TNF may be advantageously combined with predetermined amounts of other proteins such as lymphotoxin and/or interferon.

Glycosylated tumor necrosis factor also includes multimeric forms. TNF spontaneously aggregates into multimers, usually dimers or higher multimers. Multimers are cytotoxic and accordingly are suitable for use in *vivo* therapy. While it is desirable to express and recover glycosylated TNF as a substantially homogeneous multimer or monomer, glycosylated TNF may be used therapeutically as a mixture of different multimers.

Derivatives of glycosylated TNF are included within the scope of the present invention. Derivatives include covalent or aggregative conjugates with other chemical moieties. Covalent derivatives are prepared by linkage of functionalities to groups which are found in the glycosylated TNF amino acid side chains or at the N– or C- terminal, by means known in the art. These derivatives may, for example, include aliphatic esters or amides of the carboxy terminus or residues containing carboxyl side chains; O-acyl derivatives of hydroxyl group-containing residues; and N-acyl derivatives of the amino terminal amino acid or amino-group containing residues, such as lysine or arginine. The acyl group is selected from the group of alkyl moieties, including C_3 to C_{10} normal alkyl, thereby forming alkanoyl species, and carbocyclic or heterocyclic compounds, thereby forming aroyl species. The reactive groups

preferably are difunctional compounds known for use in cross-linking proteins to insoluble matrices through reactive side groups. Preferred derivatization sites are at cysteine and histidine residues.

Covalent or aggregative derivatives are useful as reagents in immunoassay or for affinity purification procedures. For example, glycosylated TNF is insolubilized by covalent bonding to cyanogen bromide-activated Sepharose by conventional methods or adsorbed to polyolefin surfaces, with or without glutaraldehyde cross-linking for use in the assay or purification of anti-TNF antibodies, cell surface receptors, or soluble extracellular portions thereof. Glycosylated TNF may also be labeled with a detectable group, e.g., radioiodinated by the chloramine T procedure, covalently bound to rare earth chelates or conjugated to another fluorescent moiety for use in diagnostic assays, especially for diagnosis of glycosylated TNF levels in biological samples by competitive-type immunoassays. Such derivatives need not exhibit cytotoxic activity, only cross-reactivity with anti-TNF.

DNA which encodes glycosylated TNF can be obtained by chemical synthesis, by screening reverse transcripts of mRNA from peripheral blood lymphocytes or cell line cultures, or by screening genomic libraries from any cell. Glycosylated TNF is synthesized in host CHO (or other eukaryotic) cells transformed with vectors containing DNA encoding TNF. A vector is a replicable DNA construct. Vectors are used either to amplify DNA encoding TNF and/or to express DNA which encodes TNF. An expression vector is a replicable DNA construct in which a DNA sequence encoding TNF is operably linked to suitable control sequences capable of effecting the expression of TNF in CHO host cells. Such control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control termination of transcription and translation.

Vectors comprise plasmids, viruses (including phage), and integrable DNA fragments (i.e., integratable into the CHO genome by recombination). Once it

has transformed a CHO host, the vector replicates and functions independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, “vector” is generic to "plasmid". However, all other forms of vectors which serve an equivalent function and which are, or become, known in the art are suitable for use herein. Suitable vectors contain replicon and control sequences which are derived from species compatible with the CHO host cells. Transformed host cells are CHO or other eukaryotic cells which have been transformed or transfected with tumor necrosis factor vectors constructed using recombinant DNA techniques. Transformed CHO or other eukaryotic host cells ordinarily express TNF.

DNA regions are operably linked when they are functionally related to each other. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence. A ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation. Generally, but not necessarily, operably linked means contiguous, and, in the case of secretory leaders, contiguous and in reading phase.

Vectors must contain a promoter which is recognized by the host organism. This is generally a promoter homologous to the intended host.

Expression vectors for CHO cells ordinarily include, if necessary, an origin of replication, a promoter located upstream from the gene to be expressed, along with a ribosome binding site, RNA splice site (if intron-containing genomic DNA is used), a polyadenylation site, and a transcriptional termination sequence. The transcriptional and translational control sequences in expression vectors used in transforming CHO cells are often provided by viral sources. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and Simian Virus 40 (SV40). The early and late promoters are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin or replication. Smaller or larger SV40 fragments may also be used, provided the approximately 250

bp sequence extending from the Hind III site toward the Bgl I site located in the viral origin of replication is included. Further, it is also possible, and often desirable, to use human genomic promoter, control, and/or signal sequences normally associated with TNF.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adenovirus, VSV, or BPV source), or may be provided by the CHO cell replication mechanism. If the vector is integrated into the CHO host cell chromosome, the latter is often sufficient.

While CHO cells are disclosed as the preferred host cell it is expected that TNF-α recombinantly produced in any eukoryotic host cell system will be glycosylated in the same manner as the TNF-α obtained from CHO cells. Thus, for providing glycosylated human TNF-α, any suitable eukaryotic host cell system may be used.

Glycosylated TNF initially is recovered from cultures media. Transformed nonsecreting cells are lysed by sonication or other acceptable method and debris separated by centrifugation, while the supernatants from secreting cells (such as induced cell lines) are simply separated from the cells by centrifugation. Then, any one or more of the following steps may be used, or other methods entirely may be substituted. The following method was used to purify glycosylated TNF to a degree sufficient for sequencing. This is not necessarily coextensive with the purification required for a therapeutic product.

As an initial purification step, glycosylated TNF is adsorbed onto a hydrophobic substance from the lysed culture of supernatant culture medium. The hydrophobic substance preferably is a nongelatinous hydrophobic surface such as a silicate or polyolefin, although alkyl Sepharose is also suitable. The preferred embodiment is controlled pore glass. A ratio of about one volume of controlled pore glass is mixed with 50 volumes of supernatant and the adsorption allowed to proceed at about 4°C without agitation over a period of about 30 minutes to two hours,

preferably about one hour, under slightly alkaline conditions. The adsorbent generally should thereafter be washed with a suitable buffer to remove entrapped contaminant proteins.

The adsorbed glycosylated TNF is eluted from the hydrophobic substance by altering the solvation properties of the surrounding medium. The elution can be accomplished by passing a solution buffered at approximately pH 7 to 8.5, preferably around 8, containing 1M salt and an effective amount of an aqueous solution of a water miscible organic polyol, such as, for example, ethylene glycol or glycerin, ordinarily ethylene glycol in the range of 10-30 percent v/v, preferably around 20 percent v/v. Of course, the optimum conditions will depend upon the polyol which is used. The glycosylated tumor necrosis factor-containing elution fractions are detected by any conventional assay.

Further purification may be obtained by adsorption of TNF onto a tertiary or quaternary amino anion exchange resin. The preferred resins for this purpose are hydrophilic matrix resins such as cross-linked polystyrene, dextran or cellulose substituted with alkyl tertiary or quaternary amino groups.

Purification to substantial homogeneity is achieved only upon further separation on SDS-PAGE electrophoresis or C4-reverse phase high pressure liquid chromatography. This product, however, may not be desirable for therapeutic use, because it may lose substantial activity upon exposure to SDA or HPLC organic solvent.

Glycosylated tumor necrosis factor prepared according to the present invention is prepared for administration by mixing the glycosylated TNF having the desired degree of purity with physiologically acceptable carriers, i.e., carriers which are nontoxic to recipients at the dosages and concentrations employed. Ordinarily, this will entail combining the glycosylated TNF with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose or dextrins, chelating agents such as EDTA, and other stabilizers and excipients. The carrier should be formulated to stabilize the glycosylated TNF as a dimer and/or preferably, a trimer. This is accomplished by

avoiding salts or detergents in concentrations that dissociate tumor necrosis factor into monomers. Alternatively, conditions that aggregate glycosylated TNF into higher multimers should also be avoided. Generally a nonionic surfactant such as TWEEN 20 is employed to eliminate excessive aggregation during purification as well as lyophilization or aqueous storage. Glycosylated TNF to be used for therapeutic administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes. Glycosylated TNF ordinarily is stored in lyophilized form, or may simply be frozen.

Human TNF is known to be useful in the treatment of many human diseases and conditions, either alone or with other active principles, such as interferon, or inactive carriers, diluents or excipients. The glycosylated human TNF of the present invention may be substituted for the non-glycosylated recombinantly-produced TNF in any of such applications and will provide increased half-life in body fluids, improved binding to receptors, and protection against the influence of proteases. Examples of such indications include solid tumors, such as melanoma, EL-4 lymphoma, Meth-A tumors, melanocytes, carcinomas of the liver or lung, metastases or mammary carcinomas, and cancers including breast, anal, colorectal, head, neck and other body organs, leukemia, viral infections, cachexia, etc. Additionally, glycosylated human TNF can be used to prevent or mitigate parasitic, bacterial, and viral infections, as well as prevention of autoimmune diseases. The glycosylated human TNF of the present invention may be formulated and administered as an injection, eye drop, nasal drop, inhalant, external preparation, oral preparation, rectal preparation, or vaginal preparation. The daily doses of the glycosylated recombinant human TNF may generally be in the range of from 50 to about 100,000,000 units. The daily dose may be increased or decreased according to the direction for use and symptoms of the recipient.

The amount of glycosylated recombinant human tumor necrosis factor that is administered depends, for example, upon the route of administration, the condition in question, and the condition of the patient. Accordingly, it will be

necessary for the therapist to titer the dosage and modify the route of administration as required to obtain optimal activity for the patient. Such modification can be effected by one skilled in the art without undue experimentation.

The glycosylated human recombinant TNF prepared according to the present invention is particularly useful because, compared to non-glycosylated human TNF, the glycosylated TNF may have an increased half-life in body fluids, improved binding to receptors, and may be better protected against the influence of proteases. Accordingly, it should be understood that the glycosylated TNF-α of the present invention may be used for every indication in which prior art non-glycosylated TNF-α is currently known. Those of ordinary skill in this art will understand that the known protcols for formulation, administration and dosage for non-glycosylated TNF-α may be initially used for the glycosylated TNF-α of the present invention subject to experimental modification in light of the expected increased half-life and improved efficiency of the glycosylated form.

Glycosylated TNF can optionally be combined with other antineoplastic agents such as chemotherapeutic antibiotics such as actinomycin-D, adriamycin, aclacinomycin A, or with agents to augment or stimulate the immune response, such as immunoglobulins such as gamma globulin, including immunoglobulins having affinity for the cell surface antigens of neoplasms. In addition, since interferons act synergistically with TNF in cell lysis assay, alpha, beta or gamma interferon is desirably combined with glycosylated TNF composition or glycosylated TNF and lymphotoxin-containing composition. A typical formulation comprises glycosylated TNF and gamma interferon in a unit activity proportion of from about 0.1:1 to 200:1, ordinarily 10:1, and may contain lymphotoxin in place of a portion of the glycosylated TNF. These proportions, of course, are subject to modification as required by therapeutic experience.

Glycosylated TNF compositions may be administered to any tumor-bearing animals. The route of administration is in accord with known methods, e.g., intravenous, intraperitoneal, intramuscular, intralesional infusion or injection of

sterile glycosylated TNF solutions, or by timed release systems as noted below. Glycosylated TNF may be administered intralesionally, i.e., by direct injection into solid tumor. In the case of disseminated tumors such as leukemia, administration is preferably intravenous or into the lymphatic system. Tumors of the abdominal organs such as ovarian cancer are advantageously treated by intraperitoneal infusion using peritoneal dialysis hardware and peritoneal-compatible solutions. Ordinarily, however, glycosylated TNF is administered continuously by infusion, although bolus injection is acceptable.

Glycosylated TNF can also be administered from an implantable timed-release article. Examples of suitable systems for proteins having the molecular weight of glycosylated TNF dimers or trimers include copolymers of L-glutamic acid and gamma ethyl-L-glutamate, poly(2-hydroethyl-methacrylate) or ethylene vinyl acetate. Such an article is implanted at surgical sites from which tumors have been excised. Alternatively, glycosylated TNF is encapsulated in semipermeable microcapsules or liposomes for injection into the tumor. This mode of administration is particularly useful for surgically inexcisable tumors such as brain tumors.

The amount of glycosylated TNF that is administered will depend, for example, upon the route of administration, the tumor in question and the condition of the patient. Intralesional injections will require less glycosylated TNF on a body weight basis than will intravenous infusion, while some tumor types, e.g., solid tumors, appear to be more resistant to glycosylated TNF than other, e.g., leukemic. Accordingly, it is necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal cytotoxic activity towards the target tumor, as can be determined, for example, by biopsy of the tumor or diagnostic assays for putative cancer markers such as carcinoembryonic antigen, in view of any recombinant toxicity encountered at elevated dosage.

Example 1: Production of Recombinant Human TNF from Chinese Hamster Ovary Cells

A clone expressing recombinant human TNF made in the manner described in Korn et al, supra, the entire contents of which are hereby incorporated by reference, was obtained from the laboratory of Prof. D. Wallach, Weizmann Institute of Science, Rehovot, Israel. The clone was propagated in Dulbecco Modified Eagle's Medium (DMEM) supplemented with proline, glutamine and 10% fetal bovine serum until production started. Production was carried out in a one-liter spinner with disc carriers. The spinner was seeded with 2.5 x 10^9 cells, which were maintained in serum-supplemented medium for five days, after which the medium was changed to production medium (DMEM, ADC-1 supplement, Beit Haemck Industries, Israel), 0.2 μg per ml insulin and 0.5 μg protein per ml serum active fraction. Production was carried out in serum-free medium. Two harvests were collected every day and the spinner was kept running in production for three months. Crude concentrated recombinant human TNF was purified on an affinity column which contained recombinant human tumor necrosis binding protein coupled to Fractogel TSK-AF-CDI resin, a resin manufactured by Toso-Haas.

More specifically, the glycosylated human TNF is purified using the following steps:

1. Clarification and concentration
2. Affinity chromatography column
3. Concentration, dialysis to the final buffer and terminal filtration

Filtration and Concentration

Recombinant human TNF crude harvest was clarified on a 1.2 micron filter and then concentrated 20-fold in the Pellicon system with 10 kDa cut-off membrane. The concentrated crude harvest was maintained at -70°C until use. Before being loaded onto the column, the thawed crude was filtered through 0.2 micron filter

in order to remove microorganisms and particles which were created as a result of the freezing/thawing action.

In general, it is not recommended to concentrate crude glycosylated TNF more than 20-fold, since further concentration of the 2% serum proteins might damage the biologically active protein in the crude. However, this limitation is irrelevant for the present invention, since the TNF is produced in low protein content serum-free medium.

Affinity Chromatography Column

Crude concentrated recombinant human TNF was purified on an affinity column which contains recombinant human TNF Binding Protein-1 (TBP-1), the TNF receptor, soluble p55 coupled to 56 ml of Fractogel TSK-AF-CDI resin (Merck). The recombinant human TNF binding protein was chosen for this purpose based upon the high association constant of TNF to its soluble receptor, TBP-1. The coupling procedure involved overnight shaking of 4.5 mg/ml concentrated recombinant human TBP-1 in 0.1 M phosphate buffer (pH 7.5) with the resin. The binding ratio is 7-8 mg recombinant human TBP-1 per ml resin. Note that one gram of resin is self-rising to 3.5 ml when suspended with the reaction liquid.

TBP-CDI Column

The chromatography procedure consisted of loading the crude onto the column which had been pre-equilibrated with PBS, washing the 30 bed volumes of HSPB and washing back to PBS (30 bed volumes) before the start of the elution. The elution was carried out with 100 mM NaCl in 50 mM citric acid (pH 2.5). The elution main peak was cut at 65% of its way down and collected into 2 M Tris (pH 8.5) for titration of the acidic pH (which is destructive to TNF activity) to pH 8. The titration was performed in a ratio of 400 microliters Tris per 5 ml of elution buffer. After the elution peak returned to baseline, the column was regenerated with 10 bed volumes of

50 mM citric acid without NaCl and then regenerated with 20 bed volumes of HSPB. The following flow chart lists all of the column's run steps:

TBP 1-CDI COLUMN AFFINITY CHROMATOGRAPHY

Equilibration with PBS

↓

Load and wash with PBS

↓

Wash with HSPB

↓

Wash with PBS

↓

Elution with 0.1 M NaCl in 50 nM Citric Acid (pH2.5)
into untitrated Tris base

↓

Column regeneration with 50 mM Citric Acid

↓

Regeneration with HSPB

Note: In this production campaign, the PBS wash before elution was omitted.

At the end of the chromatography cycle, the column was washed with 20 bed volumes of 1 M NaCl and 0.05% NaN_3 and maintained in this buffer at 4°C.

Table 1 shows a typical distribution of the recombinant human TNF through the columns fractions as measured by spectrophotometric measurement (A_{280}/EC) and bioassay.

TABLE 1

FRACTION	VOLUME (ml)	A_{280}	A_{280}/EC (mg/ml)	A_{280}/EC TOTAL (mg)	BIO ASSAY (MU/ml)	BIO ASSAY (mg/ml)	BIO ASSAY TOTAL (mg)
Input	900	N.D.	-	-	1.47×10^{6}	0.0036	33
El-2	138	0.213	0.173	23.8	5.59×10^{6}	0.127	18.9

Concentration and Dialysis

The elution fractions from several chromatography cycles were pooled together and concentrated in a Filtron Minisette system with 10kDa cutoff membrane to a concentration of 1.65 mg/ml according to absorbance (A280 divided by the extinction coefficient, A280/EC). The concentrated pool was dialyzed against 20mM Tris-CHl; 100mM NaCl (pH 8.0).

Material Distribution Throughout the Columns

A summary of the material balance results is presented in the tables below.

Table 2 shows a typical yield of the CDI-TBP-1 affinity column.

TABLE 2

TEST METHOD/ PARAMETER	(A_{280}/EC)/BIOASSAY	BIOASSAY
RECOVERY (%)	72.9	57.3

Table 3 shows a typical distribution for the r-hTNF through the column's fractions in the reprocess chromatography, as measured by A280/EC and Bioassay.

TABLE 3

FRACTION	VOLUME (ml)	A_{280}	A_{280}/EC (mg/ml)	A_{280}/EC TOTAL	BIO ASSAY (MU/ml)	BIO ASSAY (mg/ml)	BIO ASSAY TOTAL (mg)
Input	130	0.191	0.156	20.28	4.88	0.119	15.47
U.B.	200	U.L.			N.D.		
HSPB-1	215	U.L.			N.D.		
EL-1	80	U.L.			N.D.		
EL-2	120	0.146	0.119	14.28	3.82	0.094	11.28
EL-3	130	U.L.			N.D.		
EL pH 2.5	135	0.003	0.002	0.27	N.D.		
50 mM citric	50	0.005	0.004	0.20	N.D.		
HSPB II	125	0.048	0.039	4.87	N.D.		

* N.D. = Not Done

** U.L. = Under LOD = Under limit of detection

Table 4 shows a typical yield and column balance summary.

TABLE 4

TEST METHOD/ PARAMETER	(A_{280}/EC)/ BIOASSAY	BIOASSAY
RECOVERY (%)	70.4	72.9
BALANCE	96.7	N.D.

* N.D. = Not done

Pure recombinant human TNF was tested by six analytical techniques: carbohydrate composition, carbohydrate mapping, gel electrophoresis (SDS-PAGE), isoelectric focusing, capillary electrophoresis, and reverse phase high performance liquid chromatography.

Monosaccharide composition

Aliquots of recombinant human TNF prepared as above were hydrolyzed using three different sets of conditions:

(1) Release of amino sugars with 4M HCl;

(2) Release of neutral sugars with 2M trifluoroacetic acid;

(3) Release of sialic acid with 0.1M HCl.

The released monosaccharides were separated on the Dionex system. The system is comprised of a pump, pulsed electrochemical detector, autosampler, and an anion exchange column. An isocratic elution using 16 mM NaOH was used for the separation, identification and quantitation of the neutral and amino monosaccharides. Sialic acid was separated on the same anion exchange column with a gradient of 30-990 mM sodium acetate in 100 mM NaOH.

Oligosaccharide Mapping

Chemical release of oligosaccharide was performed on a GlycoPrep-1000 system from Oxford GlycoSystems. The GP-1000 is a bench-top chemical reactor which automatically cleaves the glycosidic bond with anhydrous hydrazine. The GP-1000 can operate in three modes: N, N+O and O. These modes give a preferential release of N-linked, O-linked or both, through lower recoverY (about 85%).

Two aliquots were hydrazinolyzed by the GP-1000, one in the O-mode (Fig. 1A) and a second in the N+O mode (Fig. 1B). The released oligosaccharides were separated and identified by the Dionex system with a PA-100 column.

Gel Electrophoresis (SDS-PAGE)

A 20% acrylamide SDS gel using a tall Mighty Small apparatus (Hoeffer) was used to separate and compare recombinant human TNF samples from Chinese hamster ovary cells, as well as from *E. coli*, and aliquots of TNF incubated with enzymes. The gel was loaded with 3 micrograms protein of each sample and stained for 30 minutes with Coomassie R-250.

Isoelectric Focusing

The separation of charged glycoforms was effected using 0.25 mm thick gel in a 13 x 26 cm. casting unit. The gel composition was 2.6% acrylamide, 4% ampholytes, and 10% glycerol. Five micrograms of each sample were loaded. The gel was run for 5000 Volt-hours, using 0.2 M NaOH as cathode solution and 0.2 M H3PO4 as anode solution.

Capillary Electrophoresis

TNF samples were separated by capillary zone electrophoresis on an ABI Capillary Electrophoresis system (270A-HT, Applied Biosystems). The electrophoresis conditions were as follows:

Buffer: 100 mM phosphate (pH 2.5)

Capillary: 72 mm length capillary

Detection: 200 nm

Voltage: 20 kV

Reverse Phase High Performance Liquid Chromatography

The chromatographic conditions used in this test were based on those described in European Patent 168214. The chromatography was conducted at room temperature using a C4 RP-HPLC column, 25cm x 4.6 mm, and eluted with a gradient of 1-propanol in 0.1% trifluroacetic acid. Detection was at 280 nm on a UV detector.

Results

Carbohydrate Composition

The results from the monosaccharide composition assayed on the Doinex system indicated that galactose amine, galactose, and sialic acid were present in the TNF molecule produced according to the present invention. The ratio of 1:1:1.5 was obtained and reconfirmed. This ratio and composition indicates an O-linked oligosaccharide.

Carbohydrate Mapping

In order to demonstrate that the recombinant TNF produced from Chinese hamster ovary cells is a glycoprotein with O-linked oligosaccharide, a chemical release of the

oligosaccharide was performed using the GlycoPrep-1000. The released oligoforms were separated by high performance anion exchange pulsed electrochemical detector on the Dionex system. The oligomap results obtained are shown in Figures 1A and 1B. In the GlycoPrep-1000 "O-mode" (Fig. 1A), only one peak of oligosaccharide was detected, eluting in the chromatogram at the same time as O-linked oligosaccharide standards run separately (data not shown). The released oligosaccharide in the "N+O" mode (Fig. 1B) gave the same chromatogram with no additional peaks, indicating the

absence of any known N-linked oligosaccharide. Other oligosaccharides may be present in minor amounts.

Gel Electrophoresis

TNF prepared according to the present invention was incubated with neuraminidase for two hours to remove sialic acid. An aliquot was further incubated with O-glycanase for 18 hours. From that sample, an aliquot was further incubated with N-glycanase for 18 hours. Samples were kept frozen at -70°C until analysis.

The gel obtained, (not shown) showed a major band at 17,000 Daltons, primarily TNF, and an additional band with a higher molecular weight, presumably a glycosylated form of recombinant human TNF. When incubated with neuraminidase, most of the glycosylated form turned into the 17,000 Daltons band. The gel indicated the presence of enzyme-sensitive glycoforms, affecting mainly the sialic acid and the O-linked glycans.

Isoelectric Focusing

Isoelectric focusing gels performed with recombinant TNF produced from Chinese hamster ovary cells and recombinant TNF produced from *E. coli* indicated that the major bands are similar (not shown). Extra bands observed with TNF produced in CHO cells were sensitive to enzymatic digestion.

Incubation with neuraminidase removed most of the extra bands at pI=5.85 and 5.2. The removal of the acidic charged group, sialic acid, resulted in increase in the level of the basic forms. The other enzymatic incubations did not further affect charge and, thus, did not alter migration in the isoelectric focusing analysis.

Capillary Electrophoresis

Figure 2 represents the electropherograms obtained in capillary electrophoresis assays. Figure 2A shows the results of capillary electrophoresis of recombinant human TNF from CHO cells; 2B shows the results for recombinant human TNF from CHO cells + neuraminidase; 2C shows results from recombinant human TNF from CHO cells plus neuraminidase plus O-glycanase; 2D shows the results for recombinant human TNF from CHO cells plus neuraminidase plus O-glycanase plus N-glycanase; and 2E shows the results of capillary electrophoresis analysis of recombinant human TNF produced in *E. coli*.

The profile of TNF produced by CHO cells, Figure 2A, exhibits a main peak, which is probably unglycosylated recombinant human TNF, as well as additional peaks not present in the *E. coli* recombinant human TNF, Figure 2E. The glycosylated peaks represent about 35% of the molecules.

Figure 2B demonstrates that neuraminidase removed most of the charge, sialic acid, and that O-glycanase removed the oligosaccharide core, as shown in Figure 2C. Figure 2D shows that additional incubation with N-glycanase did not alter the electropherogram.

Reverse Phase High Performance Liquid Chromatography

When recombinant human TNF prepared in CHO cells and recombinant human TNF prepared in *E. coli* were subjected to reverse phase high performance liquid chromatography, the two compounds co-eluted, indicating a resemblance in the protein core. Chromatograms of recombinant human TNF from CHO and *E. coli* showed the two proteins as co-migrating proteins.

Summary of Characterization Tests

The above assays clearly demonstrated that recombinant human TNF prepared in CHO cells has an additional form not present on recombinant human TNF obtained from *E. coli*. The form from CHO cells was found to be O-glycosylated. All of the tests performed indicated that recombinant human TNF obtained from CHO

cells is similar to recombinant human TNF from *E. coli* in the protein core, but differs in that the CHO TNF is glycosylated. The nature of the glycosylated form was determined by composition analysis, oligomap and capillary electrophoresis.

Purity Determination of TNF Bulk by SDS-PAGE Analyses

A limit testm whereby various amounts of the final TNF bulk were run on SDS-PAGE and stained by Coomassie blue. The limit of detection of HSA on the gel is 150 ng and the maximum load of TNF was 40 micrograms per lane. There was no visible impurity band in the TNF lanes. The conclusion therefore is that there is no single contaminant band which is higher than 0.37%.

In conducting these assays, samples applied on the gel were: (1) Sample buffer; (2) 10 micrograms TNF Bulk-2; (3) 20 micrograms TNF-Bulk 2; (4) 40 micrograms TNF Bulk-2; (5) 75 ng HSA; (6) 100 ng HSA; (7) 150 ng HSA; (8)LMW markers

Qualitative purity were obtained by SDS-PAGE-silver stained. In obtaining the results, 9 and 18 micrograms of the final TNF Bulk were run on SDS-PAGE and stained by silver stain. The samples applied on the gel were: (1) Sample buffer; (2) 9 micrograms TNF Bulk-1; (3) 9 micrograms TNF Bulk-2; (4) 18 micrograms TNF Bulk-2; (5) LMW markers. No impurity was visible.

TNF-Bulk Concentration

The final bulk concentration was determined by a variety of analytical test methods: Bradford, A280/E.C., ELISA and bioassay. The results are summarized in Table 5.

TABLE 5

TEST METHOD	RESULT
BRADFORD	1265 μg/ml
ELISA	1658 μg/ml
A_{280}/E.C.	1651 μg/ml
BIOASSAY	50.87 MU/ml

Size Exclusion Chromatography

Size exclusion chromatography was performed. Its purpose was to determine the purity and the molecular weight of the TNF form which is present in the final bulk. The SEC profile revealed one major peak with apparent MW by retention time of about 50 kD. It is known from the literature, and from our SDS-PAGE analysis, that the TNF molecular weight is 17 kD. The conclusion is that TNF exists in the bulk in the trimer form which is reported to be the bioactive form.

Isoelectric Focusing

Isoelectric focusing was performed. The final bulk has seven isoelectric forms in the pH range of 5-7. When r-hTNF Bulk-1 was treated with neuraminidase, it collapsed to one isoelectric form. The conclusion is that all the other forms are probably glycosylated.

Isoelectric focusing of r-hTNF Bulk samples was conducted on gel -5% total acrylamide 2.6% bis-acrylamide containing 2% ampholytes, 32% (v/v) glycerol, 10 mM GLU, 10 mM LYS, pH gradients 3-10, 15 cm wide (anode to cathode), run conditions: 15°C, 20,000 V/hr.

Samples applied on the gel were: (1) Internal marker (cytochrome C); (2) pH markers; (3) 5 microliters 0.5 mg/ml TNF Bulk-1; (4) 5 microliters 0.5 mg/ml TNF Bulk-1; (5) 5 microliters 0.5 mg/ml TNF Bulk-2; (6) 0.5 mg/ml TNF Bulk-2; (7) 0.5 microliters 1.65 mg/ml TNF Bulk-2; (1) 5 microltiers 1.65 mg/ml TNF Bulk-2; (9) pH markers.

The final bulk was checked for its amino acid composition and the results are shown in Table 6:

TABLE 6

Amino Acid Composition

	Sample	TNF - Bulk 2
	Aminolab No.	A01590894.9H-HP
	Molec. Wt.	16606.28
	Smplwt. mg	0.087
	Vol sampled, ul	60
	S.B. Vol ul	330
	InjVol ul	50
	Analysis Date 19.8.94	Diln. 7.5825E-03

Hydrolyzed by Gas Phase

No.	ACID	residues	% in sample	Amt/run, nM
2	ASX	12.25	8.490	9.73
4	THR	6.13	3.733	4.87
5	SER	12.28	6.437	9.75
6	GLX	11.12	8.644	8.83
7	PRO	10.38	6.067	8.24
8	GLY	11.47	3.941	9.11
9	ALA	13.96	5.976	11.09
10 1/2	CYS	2.19	1.361	1.74
11	VAL	15.02	8.967	11.93
13	ILE	8.85	6.031	7.03
14	LEU	18.19	12.397	14.45
15	TYR	7.35	7.225	5.84
16	PHE	4.18	3.705	3.32
17	HIS	3.34	2.756	2.65
18	LYS	6.47	4.995	5.14
20	ARG	9.86	9.273	7.83 121.55
	Total-	153.05	100.00	

N-Terminal Amino Acid Sequence

The final bulk was checked for N-terminal amino acid sequence, and the results showed a sequence known from the literature:

Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His (SEQ ID NO:1)

Endotoxins Concentration

The final bulk was checked for endotoxin concentration by the LAL test, and the results showed endotoxin concentration of less than 0.25 EU/ml, or less than 0.15 EY/mg.

The tests results for Recombinant TNF Bulk are shown in Table 7:

TABLE 7

TNF-RECOMBINANT BULK

Product:	CHO r-TNF-1	Issue Date:	4.10.94
Bulk No.:	TNF-Bulk-2	Production Date:	8.8.94

General Description	
PH	8.0
CONDUCTIVITY	12.6 mS/cm
A_{280}	2.025 A_{280}/ml
PROTEIN CONCENTRATION (A_{280}/E.C.)*	1.65 mg/ml
PROTEIN CONCENTRATION (Bradford)	1.265 mg/ml
Identity	
MOLECULAR WEIGHT (SEC)	49.89 KD
ISOELECTRIC FOCUSING (IEF)	CONFORMS with TNF Bulk-1
N-TERMINAL SEQUENCING	CONFORMS**
AMINO ACID ANALYSIS(A.A.A.)	CONFORMS with TNF Bulk-1
Potency	
BIOASSAY***	50.87 U/ml
ELISA***	1.65 mg/ml
Purities & Impurities	
% PURITIES BY SDS-PAGE	99.5%
PURITY PROFILE BY SDS-PAGE	CONFORMS with TNF Bulk-1
TBP LEVEL	LT 0.01 ng/µg
Safety	
ENDOTOXINS BY LAL	LT 0.15 EU/mg

* Extinction Coefficient = 1.226 (A_{280} per mg/ml)
** Identity to the sequence known from the literature
*** NBSB Standard

Within the scope of the present invention are fragments of glycosylated TNF or mutants of glycosylated TNF or its fragments (including mature TNF) wherein one or more amino acid residues are substituted, inserted or deleted as long as such fragments or mutants retain the biological activity of the r-hTNF-α.

The degree of amino acid sequence homology which brings a polypeptide within the scope of the definition of glycosylated tumor necrosis factor fragments or mutants herein will vary depending upon whether the homology between the candidate protein and glycosylated tumor necrosis factor falls within or without the tumor necrosis factor regions responsible for cytotoxic activity. Domains which are critical for cytotoxic activity should exhibit a high degree of homology in order to fall within the definition, while sequences not involved in maintaining glycosylated TNF conformation or in effecting receptor binding may show comparatively low homology. In addition, critical domains may exhibit cytolytic activity and yet remain homologous as defined herein if residues containing functionally similar amino acid side chains are substituted. Functionally similar refers to dominant characteristic of the side chains such as basic, neutral or acid, or the presence or absence of steroid bulk. However, tumor necrosis factor as defined herein specifically excludes lymphotoxin, or TNF-beta, as well as glycosylated TNF of other species such as murine TNF-alpha.

A significant factor in establishing the identity of a polypeptide as glycosylated TNF is the ability of antisera which are capable of substantially neutralizing the cytolytic activity of mature glycosylated TNF to also substantially neutralize the cytolytic activity of the polypeptide in question. However, it should be recognized that immunological identity and cytotoxic identity are not necessarily coextensive.

Glycosylated tumor necrosis factor as defined herein includes, for example, TNF derivatives like insertion mutants, deletion mutants, or fusion proteins described above. These derivatives will bring glycosylated TNF outside of the

molecular weight established for native human glycosylated TNF. Similarly, glycosylated TNF may be engineered in order to reduce or eliminate susceptibility to hydrolysis by trypsin or other proteases.

The language "capable" of cytotoxic activity for *in vivo* tumor necrosis means that the term tumor necrosis factor includes polypeptides which can be converted, as by enzymatic hydrolysis, from an inactive state analogous to a zymogen to a polypeptide fragment which exhibits the desired biological activity. Typically, inactive precursors will be fusion proteins in which mature glycosylated TNF is linked by a peptide bond at its carbonyl terminus to a human protein or fragment thereof. The sequence at this peptide bond or nearby is selected so as to be susceptible to proteolytic hydrolysis to release glycosylated TNF, either *in vivo* or, as part of a manufacturing protocol, *in vitro.* The glycosylated TNF factor that is so generated then will exhibit the definitionally-required cytotoxic activity.

Mutant glycosylated TNF derivatives include the predetermined, i.e., site-specific, mutations of glycosylated TNF or its fragments. The objective of mutagenesis is to construct DNA that encodes glycosylated TNF as defined above, i.e., glycosylated TNF which exhibits cytotoxic activity towards tumor cells *in vitro* or acts like glycosylated TNF *in vivo*, and which retains residual homology with glycosylated TNF, but which also exhibits improved properties and activity. Mutant glycosylated tumor necrosis factor is defined as a polypeptide otherwise falling within the homology definition for glycosylated tumor necrosis factor set forth herein but which has an amino acid sequence different from that of glycosylated TNF whether by way of deletion, substitution, or insertion. For example, the lysine or arginine residues of glycosylated TNF may be mutated to histidine or another amino acid residue which does not render the protein proteolytically labile. Similarly, cysteine could be replaced by other residues and cross-linked chemically in order to confer oxidative stability. It is not necessary that mutants meet the activity requirements for glycosylated TNF, for

even biologically inactive mutants will be useful upon labeling or immobilization as reagents in immunoassays. However, in this case the mutants will retain at least one epitopic site which is cross-reactive with antibody to glycosylated TNF.

Mutations in DNA which encode the glycosylated TNF are not necessarily expressed in the final secreted product. For example, a major class of DNA substitution mutations are those in which a different secretory leader or signal has been substituted for the native human secretory leader, either by deletions within the leader sequence or by substitutions, wherein most or all of the native leader is exchanged for a leader more likely to be recognized by the CHO cells. When the secretory leader is "recognized" by the host CHO cells, the fusion protein consisting of glycosylated TNF and the leader ordinarily is cleaved at the leader-glycosylated TNF peptide bond in the events that lead to secretion of the glycosylated TNF.

Another major class of DNA mutants that are not expressed as glycosylated TNF derivatives are nucleotide substitutions made to enhance expression, primarily to avoid amino terminal loops in the transcribed mRNA or to provide codons that are more readily transcribed by the CHO, e.g., the well-known CHO preference codons for CHO expression.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice

within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

References:

Aggarwal et al, 1987 Tumour necrosis factor and related cytotoxins. Wiley, Chichester (Ciba Foundation Symposium 131) p. 39-51 (1987)

Beutler et al, Nature, 320:584-588 (1986).

Beyaert et al, FEBS Letters, 340:9-16 (1994).

Fiers, FEBS, 285:199-212 (1991).

Korn et al, Lymphokine Res., 7:349-358 (1988)

Malik et al, Eur. J. Cancer, 26:1031-1034 (1990).

Markham et al, Eur. Cytokine Netw, 6:49-54 (1995)

Marmenout et al, Eur. J. Biochem, 152:512-522 (1985)

Old, Scientific American, 258:59-60, 69-75 (1988)

Porter, TIBTECH, 9:158-162 (1991).

Vilcek et al, J.Bio.Chem., 266:7313-7316 (1991).

Wallace et al, USP 4,879,226

Aggarwal et al, EP 0168214

CLAIMS

1. Isolated biologically active glycosylated human tumor necrosis factor.

2. A method for preparing glycosylated human tumor necrosis factor, comprising:

(a) ligating DNA encoding human TNF or a physiologically active variant thereof to a replicable expression vehicle to obtain a replicable recombinant DNA comprising said DNA and said replicable expression vehicle;

(b) transforming eukaryotic cells with said replicable recombinant DNA to form transformants;

(c) culturing said transformants to cause said transformants to express said glycosylated human tumor necrosis factor; and

(d) isolating said glycosylated human tumor necrosis factor from the cultured transformants.

3. The method according to claim 2, further comprising the step of purifying the isolated glycosylated human tumor necrosis factor.

4. A method in accordance with claim 2, wherein said eukaryotic cells are Chinese hamster ovary cells.

5. A pharmaceutical composition comprising an effective amount of glycosylated human tumor necrosis factor and at least one pharmaceutically acceptable carrier, diluent, or excipient.

6. In the method for treating a human disease or condition treatable by the administration of an effective amount of human TNF alone or in combination with other active principles or inactive carriers, diluents or excipients, the improvement wherein said human TNF is biologically active glycosylated human TNF.

ABSTRACT

Glycosylated recombinant human tumor necrosis factor alpha is produced in Chinese hamster ovary cells and isolated therefrom. It may be used for the same indications as are known for non-glycosylated human TNF-α.

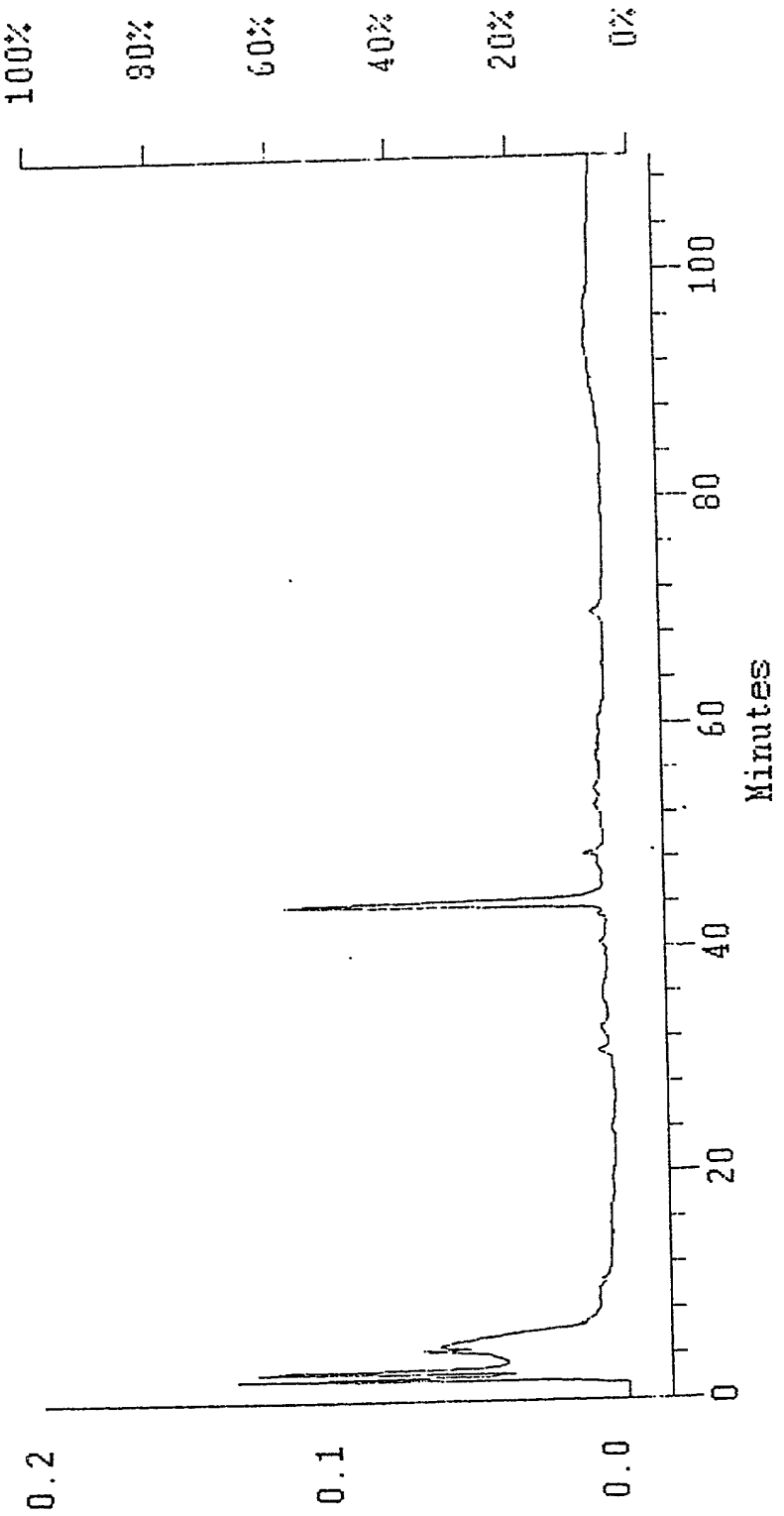

Fig. 1A

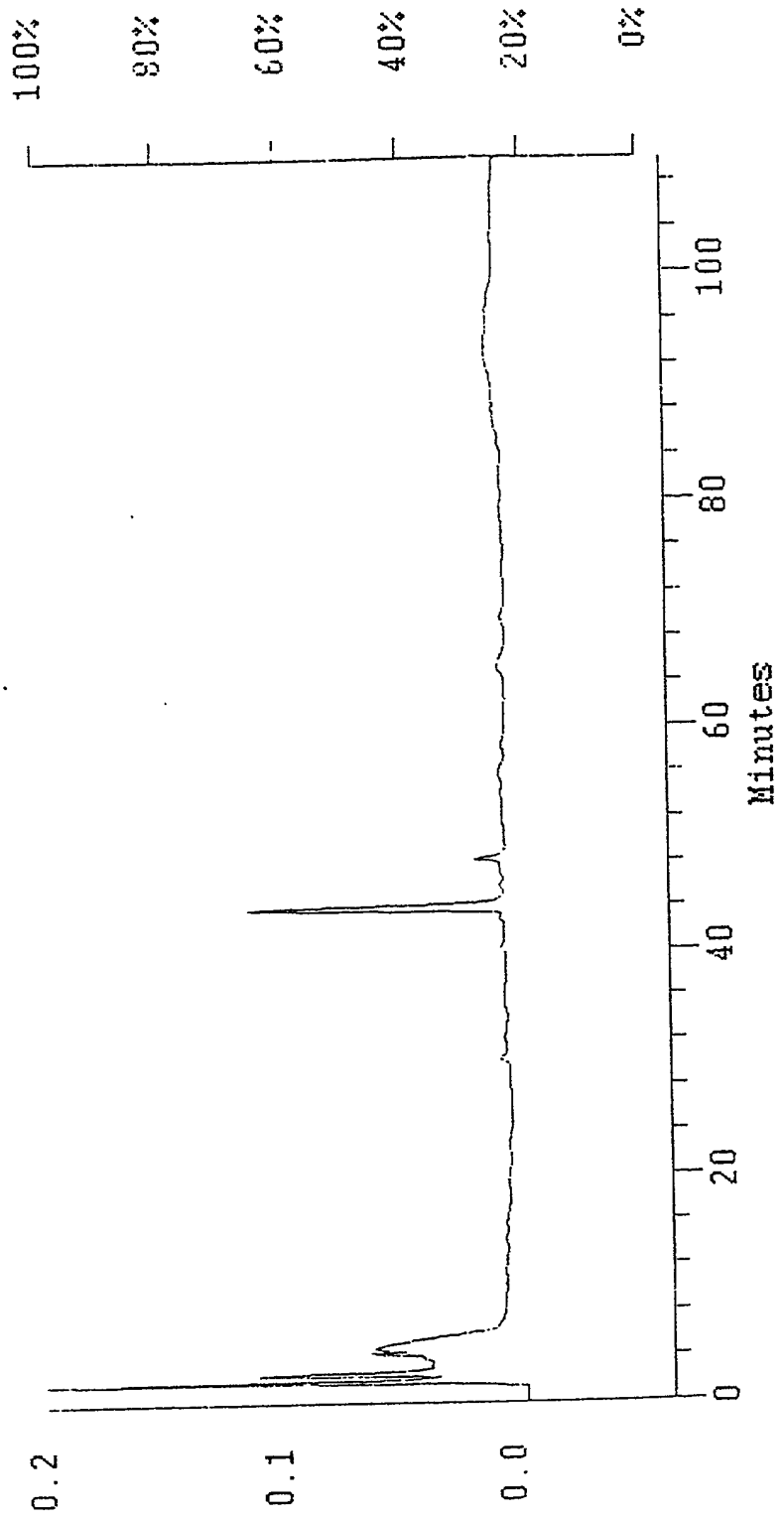

Fig. 1B

Fig. 2A

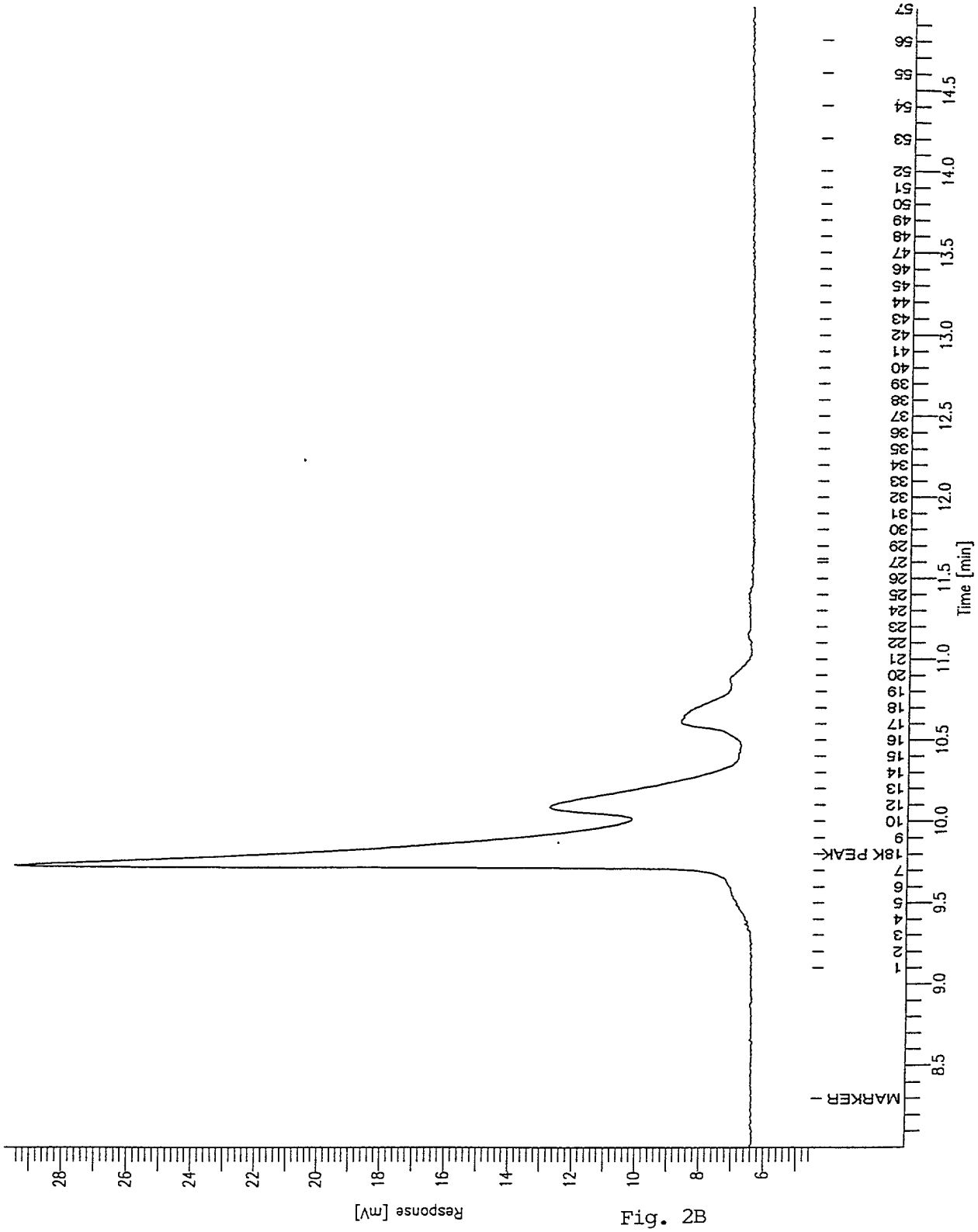

Fig. 2B

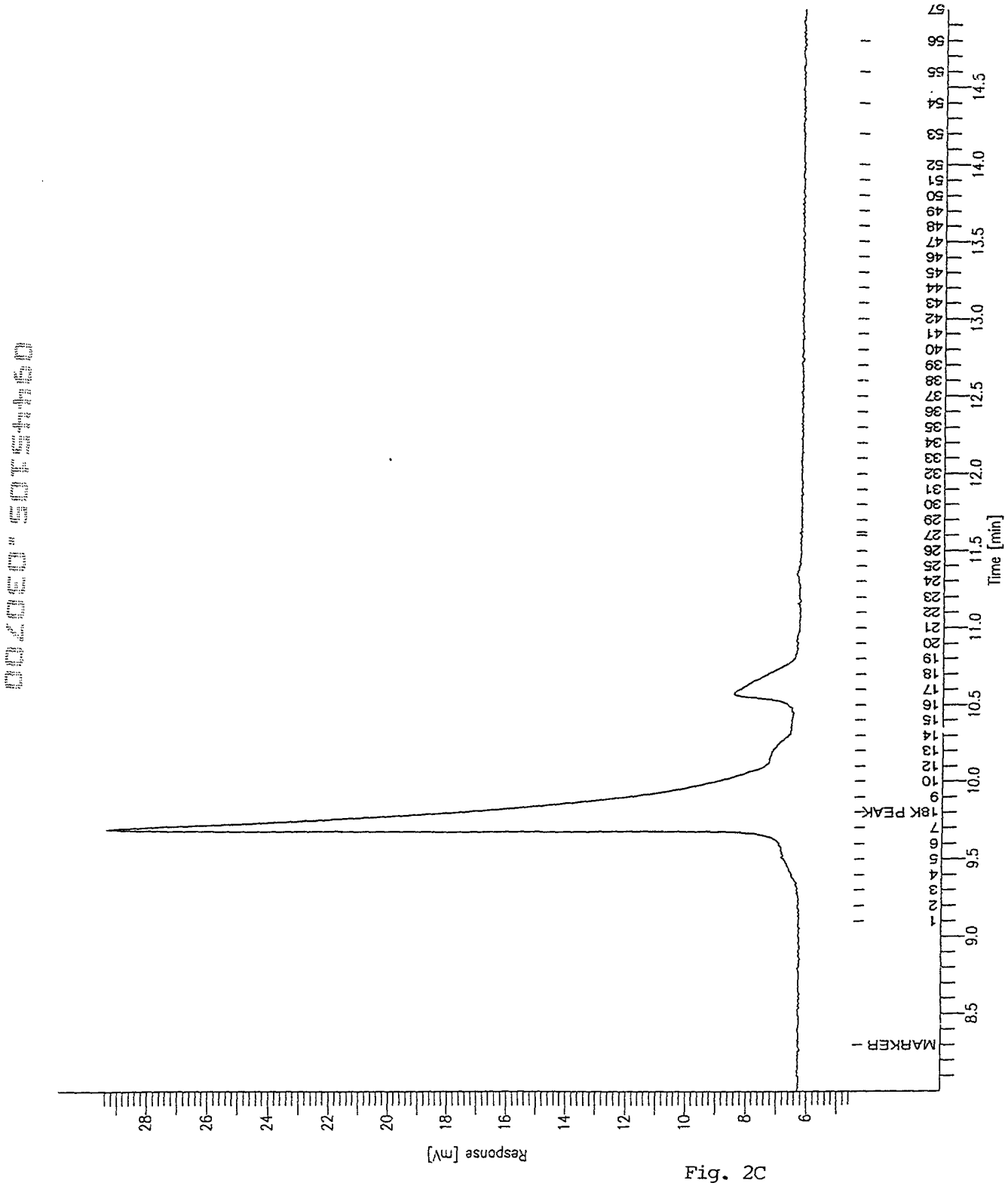

Fig. 2C

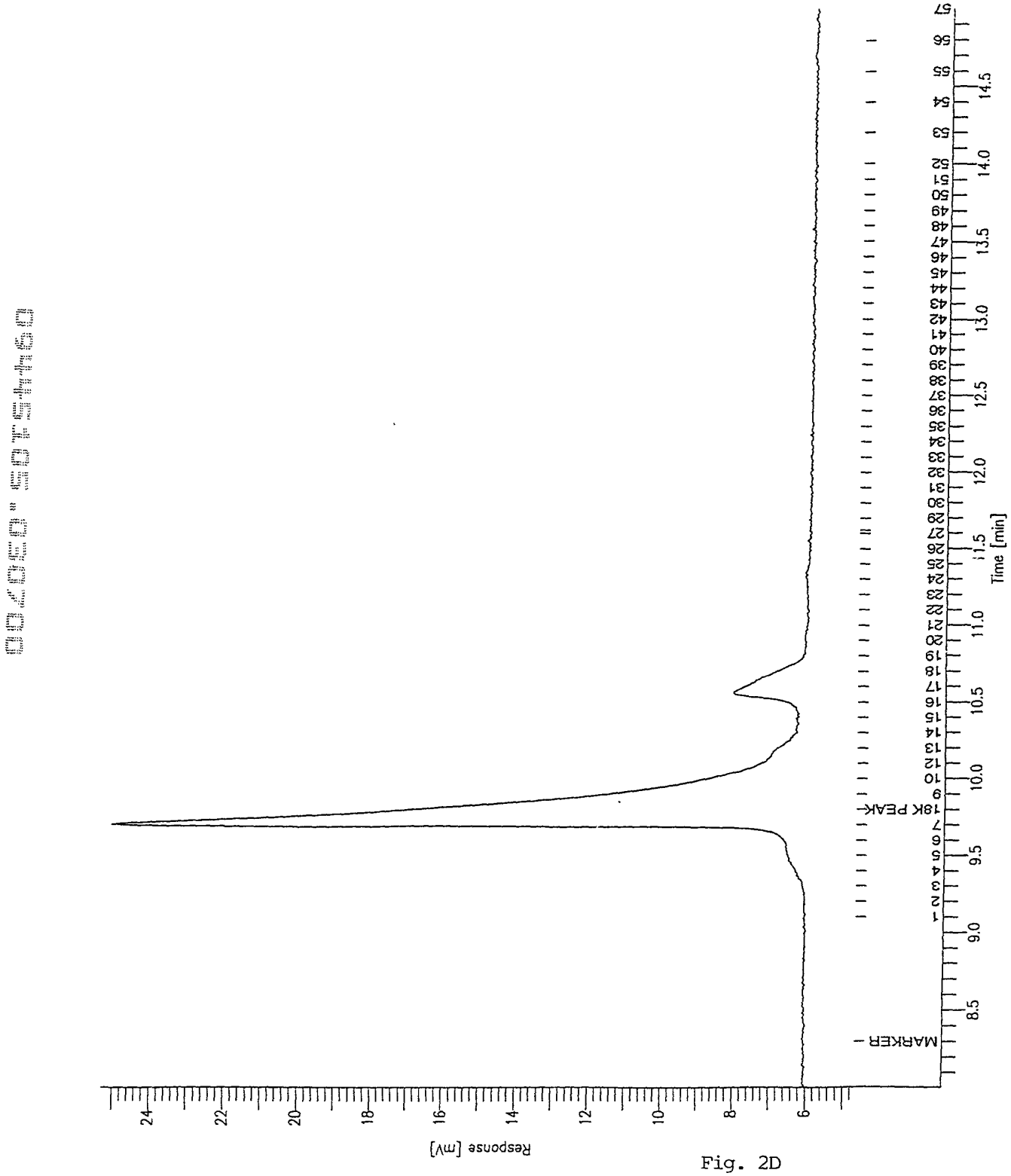

Fig. 2D

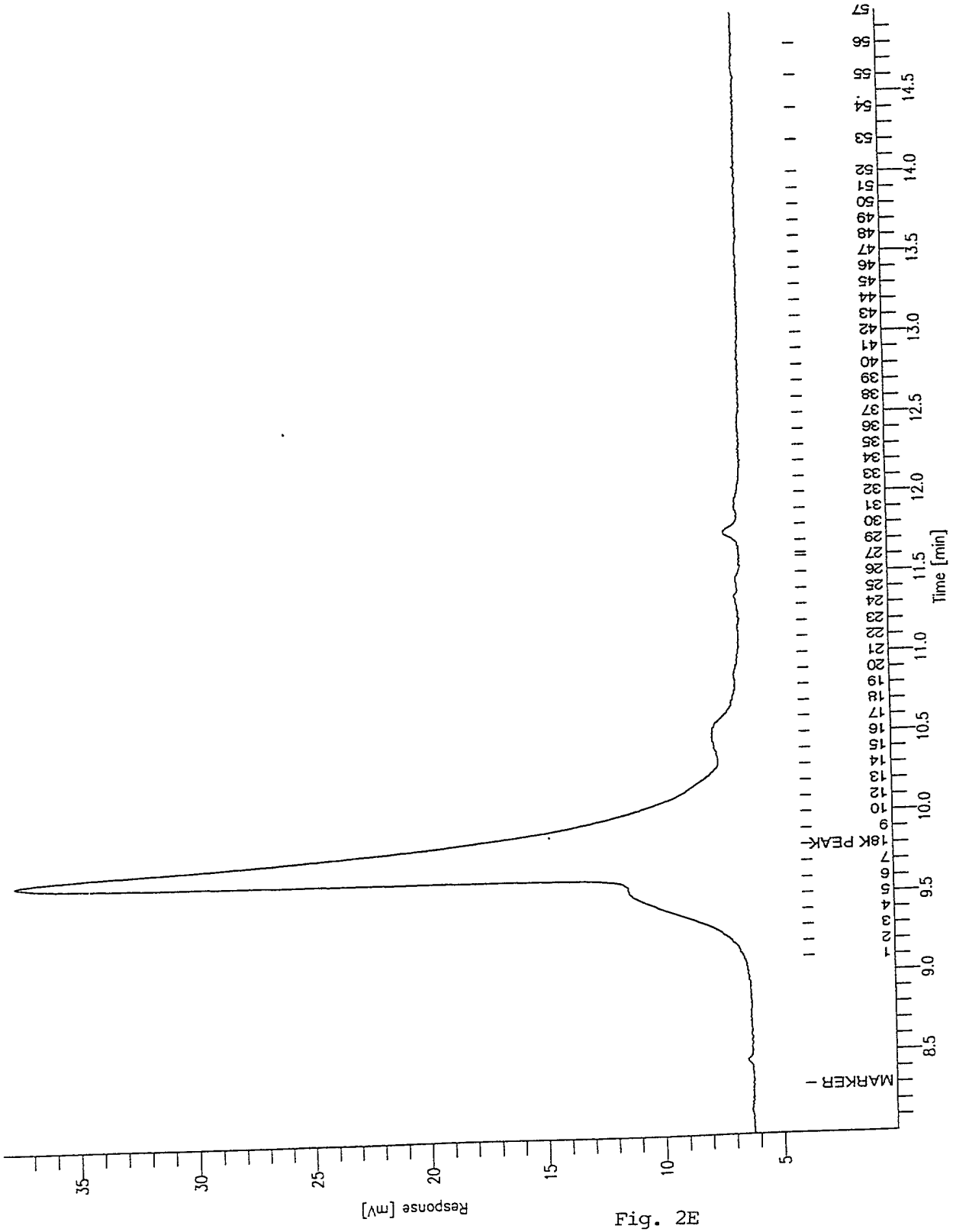

Fig. 2E

Combined Declaration for Patent Application and Power of Attorney

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PREPARATION OF GLYCOSYLATED TUMOR NECROSIS FACTOR

the specification of which (check one)

[ ] is attached hereto;

[ ] was filed in the United States under 35 U.S.C. §111 on _____, as USSN _____*; or

[XX] was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an international (PCT) application, PCT/IL98/00254; filed 01 June 1998, entry requested on _____*; national stage application received USSN _____*; §371/§102(e) date _____* (*if known), and was amended on _____ (if applicable).

(include dates of amendments under PCT Art. 19 and 34 if PCT)

I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above; and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119, 365 of any prior foreign application(s) for patent or inventor's certificate, or prior PCT application(s) designating a country other than the U.S., listed below with the "Yes" box checked and have also identified below any such application having a filing date before that of the application on which priority is claimed:

120979 (Number)	Israel (Country)	02 June 1997 (Day Month Year Filed)	[X] YES	[ ] NO
(Number)	(Country)	(Day Month Year Filed)	[ ] YES	[ ] NO

I hereby claim the benefit under 35 U.S.C. § 120 of any prior U.S. non-provisional Application(s) or prior PCT Application(s) designating the U.S. listed below, or under § 119(e) of any prior U.S. provisional applications listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information as defined in 37 C.F.R. §1.56(a) which occurred between the filing date of the prior application and the national filing date of this application:

(Application Serial No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
(Application Serial No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)

I hereby appoint the following attorneys, with full power of substitution, association, and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

SHERIDAN NEIMARK, REG. NO. 20,520 - ROGER L. BROWDY, REG. NO. 25,618 - ANNE M. KORNBAU, REG. NO. 25,884
NORMAN J. LATKER, REG. NO. 19,963 - IVER P. COOPER, REG. NO. 28,005 - ALLEN C. YUN, REG. NO. 37,971*
NICK S. BROMER, REG. NO. 33,478 - * Patent Agent

PTO CUSTOMER NO: 001444 ADDRESS ALL CORRESPONDENCE TO BROWDY AND NEIMARK, P.L.L.C. 624 9th Street, N.W., Suite 300 Washington, D.C. 20001	DIRECT ALL TELEPHONE CALLS TO: BROWDY AND NEIMARK (202) 628-5197

The undersigned hereby authorizes the U.S. Attorneys or Agents named herein to accept and follow instructions from INTERPHARM LABORATORIES LTD. as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorney or Agent and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents named herein will be so notified by the undersigned.

Title: PREPARATION OF GLYCOSYLATED TUMOR NECROSIS FACTOR
U.S. Application filed _____, Serial No. _____
PCT Application filed 01 June 1998, Serial No. PCT/IL98/00254

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF FIRST INVENTOR	INVENTOR'S SIGNATURE	DATE
Ziva MESSIKA	M. Ziva	29/11/99
RESIDENCE		CITIZENSHIP
Tel Aviv, Israel IL		Israel
POST OFFICE ADDRESS		
El Nekave Street 18, Tel Aviv 67655, Israel		
FULL NAME OF SECOND JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
Adrian HAREL	X Harel Adrian	14/11/99
RESIDENCE		CITIZENSHIP
Ness-Ziona, Israel IL		Israel
POST OFFICE ADDRESS		
Hahagana Street 5, Ness-Ziona 74022, Israel		
FULL NAME OF THIRD JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
Menachem ZE'EVI		
RESIDENCE		CITIZENSHIP
Ramat Gan, Israel IL		Israel
POST OFFICE ADDRESS		
Hibat Zion Street 6, Ramat Gan 52408, Israel		
FULL NAME OF FOURTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF FIFTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF SIXTH JOINT INVNETOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE		CITIZENSHIP
POST OFFICE ADDRESS		

ALL INVENTORS MUST REVIEW APPLICATION AND DECLARATION BEFORE SIGNING. ALL ALTERATIONS MUST BE INITIALED AND DATED BY ALL INVENTORS PRIOR TO EXECUTION. NO ALTERATIONS CAN BE MADE AFTER THE DECLARATION IS SIGNED. ALL PAGES OF DECLARATION MUST BE SEEN BY ALL INVENTORS.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Interpharm Laboratories Ltd.
(B) STREET: Science-based Industrial Park, Kiryat Weizmann
(C) CITY: Ness-Ziona
(E) COUNTRY: Israel
(F) POSTAL CODE (ZIP): 76110
(G) TELEPHONE: 972-8-9382609
(H) TELEFAX: 972-8-9407297

(A) NAME: MESSIKA, Ziva
(B) STREET: El Nekave Street 18
(C) CITY: Tel Aviv
(E) COUNTRY: Israel
(F) POSTAL CODE (ZIP): 67655

(A) NAME: HAREL, Adrian
(B) STREET: Hahagana Street 5
(C) CITY: Ness-Ziona
(E) COUNTRY: Israel
(F) POSTAL CODE (ZIP): 74022

(A) NAME: ZE'Evi, Menachem
(B) STREET: Hibat Zion Street 6
(C) CITY: Ramat Gan
(E) COUNTRY: Israel
(F) POSTAL CODE (ZIP): 52408

(ii) TITLE OF INVENTION: Preparation of Glycosylated Tumor Necrosis Factor

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: IL 120979
(B) FILING DATE: 02-JUN-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
1               5                   10                  15
```